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Engineering of Quasi-Natural *Pseudomonas putida* Strains for Toluene Metabolism through an *ortho*-Cleavage Degradation Pathway

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To construct a bacterial catalyst for bioconversion of toluene and several alkyl and chloro- and nitro-substituted derivatives into the corresponding benzoates, the upper TOL operon of plasmid pWW0 of *Pseudomonas putida* was fully reassembled as a single gene cassette along with its cognate regulatory gene, *xylR*. The corresponding DNA segment was then targeted to the chromosome of a *P. putida* strain by using a genetic technique that allows deletion of all recombinant tags inherited from previous cloning steps and leaves the otherwise natural strain bearing exclusively the DNA segment encoding the phenotype of interest. The resulting strains grew on toluene as the only carbon source through a two-step process: conversion of toluene into benzoate, mediated by the upper TOL enzymes, and further metabolism of benzoate through the housekeeping *ortho*-ring cleavage pathway of the catechol intermediate.

Many *Pseudomonas* strains are able to employ as their sole carbon source a variety of unusual chemicals, including a wide range or aromatic hydrocarbons and their derivatives (25, 30). The catabolic pathways involved (often encoded by plasmids) are composed of distinct steps, each of which can be split from the others with the tools of recombinant DNA and expressed independently (28). Not infrequently, the enzymes involved in one or more steps in a catabolic pathway have activity on structural analogs of the natural substrates and can thereby give rise to derivatives of interest for industry (29). This is the case for certain enzymes included in the catabolic pathway for toluene, *m*-xylene, *p*-xylene, and ethyl benzene, encoded by the TOL plasmid pWW0 of *Pseudomonas putida* mt-2 (14, 21). Degradation of toluene through the TOL pathway has two major steps, namely, bioconversion of this substrate into benzoate and subsequent catabolism of its aromatic ring down to Krebs cycle metabolites via a catechol intermediate that is cleaved in *meta* by one of the pathway enzymes. The enzymes that determine conversion of toluene to benzoate are encoded by the so-called upper pathway (Fig. 1), and they happen to have activity not only on the hydrocarbon but also on a variety of chloro and nitro derivatives (1–3). Therefore, the expression of the collective enzymes of the upper pathway away from the remaining TOL genes is predicted to yield a collection of nitro- and halo-substituted benzoates. However, the development of a bacterial catalyst for these biotransformations has been impossible because of the difficulty in reassembling the entire upper TOL operon along with its cognate regulatory gene (*xylR*; 21), away from the *meta*-cleavage activity.

A more general problem of using recombinant strains in biotransformations or bioremediation of aromatic hydrocarbons is the instability of the cloned genes (frequently borne by plasmids) and the inheritance of marker genes (antibiotic or not antibiotic) used for selection (4, 5). The presence of antibiotic resistance genes in strains destined for environmental release is particularly undesirable under U.S. and European Union regulations (5). While the problem of stability has been alleviated by the use since 1990 of Tn*5*-derived transposon vectors for stable chromosomal insertion of cloned genes (6, 7, 16), the resulting strains still inherit DNA segments unrelated to the desired phenotype (i.e., those of the selection markers included in the transposon vector). As a consequence, the resulting biocatalysts are still quite different from those which could arise during natural evolution, by processes involving exclusively shuffling of DNA segments through transposition (27, 30) or DNA slippage (15).

In this work, we have employed transposon vectors with excisable selection markers (18) for insertion of the reconstructed and fully functional upper TOL pathway into the chromosome of *P. putida* KT2442 (7). The resulting strains convert toluene into benzoate and further metabolize this compound through the housekeeping *ortho*-ring cleavage pathway of the catechol intermediate. Due to the loss of recombinant markers during the construction process, these genetically engineered strains differ minimally from their nonrecombinant counterparts.

Reconstruction of the upper TOL operon as a single catabolic segment. A gene cassette bearing the complete upper operon along with its native toluene-responsive regulator gene, *xylR*, was constructed by using the strategy shown in Fig. 2. The origin of the upper TOL sequences was plasmids pED3306 and pRL4, which span overlapping segments of the operon (Fig. 2). pED3306 (22) consists of pBR322 containing a 10-kb *Hin*dIII fragment (D fragment) with *xylUWCMA* (31) and the adjacent upstream region. pRL4 (kindly provided by S. Harayama, Kamaishi City, Japan) contains a 7.1-kb *Bam*HI fragment generated upon insertion of Tn*5* into the TOL plasmid downstream of the upper operon. This fragment includes the *xylMABN* sequences along with an unrelated segment of Tn*5*. During early attempts to splice different portions of the upper operon, it was noticed that subcloning of a 5.8-kb *Xba*I-*Cla*I segment from pRL4 spanning *xylMABN* was impossible in a multicopy plasmid. To overcome the problem, we used a monocopy plasmid vector with an Ω element (9) in front of the cloned DNA to prevent any expression of the inserted sequences. The specialized vector pCK02 is a derivative of the low-copy-number,

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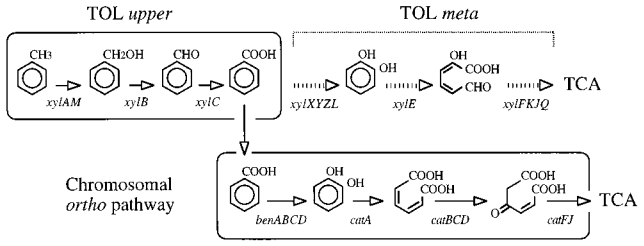

FIG. 1. Biodegradation of toluene by *P. putida* through alternative *meta* or *ortho* lower pathways. *P. putida* strains carrying the TOL plasmid pWW0 perform sequential oxidation of the CH_3 group of toluene to alcohol, aldehyde, and acid through the action of the enzymes xylene monooxygenase (encoded by *xylAM*), benzyl alcohol dehydrogenase (encoded by *xylB*), and benzaldehyde dehydrogenase (encoded by *xylC*), respectively (14, 21). The resulting benzoate is then channeled into a lower route involving *meta*-ring cleavage of the catechol intermediate through the action of the products of the TOL genes *xylXYZL*, *xylE*, and *xylFKJQ*, all the way down to Krebs cycle intermediates (TCA, tricarboxylic acid cycle). In the absence of the TOL *meta* pathway, benzoate can be channeled through an alternative, chromosomally encoded route (involving the *ben* genes) for formation of catechol (30). This is then subjected to *ortho*-ring cleavage by the product of the *catA* gene (catechol 1,2-dioxygenase) and further converted to metabolic intermediates. The *meta*-cleavage pathway is generally incompatible with the degradation of chloroaromatics (2, 30).

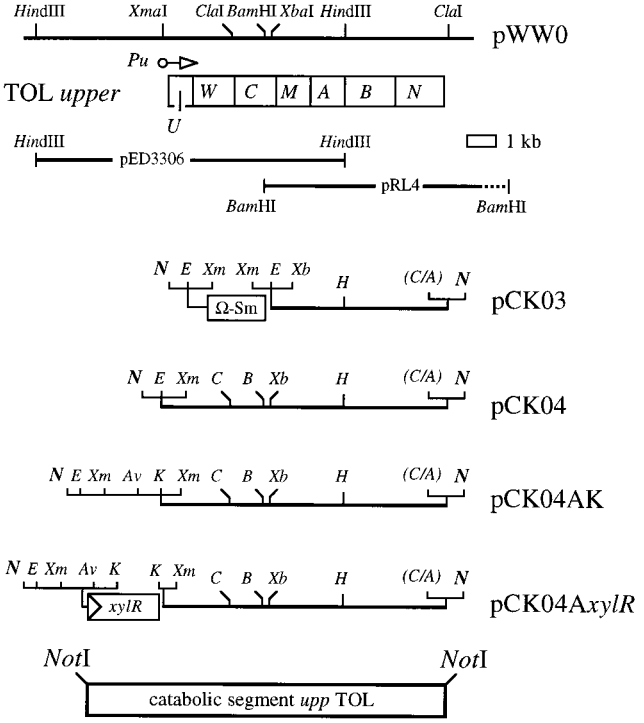

FIG. 2. Reconstruction of the upper TOL gene cluster as a single catabolic segment. The organization of the *xyl* genes of the upper TOL operon is shown at the top, aligned with the restriction map of the portion of the pWW0 plasmid involved (14, 31). Genes *xylUW* and *xylN* are not required for degradation of toluene, but they form part of the same transcriptional unit. The location and orientation of the toluene-responsive promoter of the system, *Pu*, are also indicated. *Pu* becomes activated by the XylR protein (which maps at a distant position in the pWW0 plasmid; 21) when cells encounter toluene or xylenes in the medium (1, 21). The DNA inserts of plasmids pED3306 and pRL4 used as the source of the *xyl* genes are indicated. The organization of the constructions that preceded the reassembly of the operon along with the *xylR* gene in pCK04A*xylR* is shown (see the text for an explanation). The various segments were cloned between the *Not*I sites of vector pCK01. This is a Cm^r plasmid with a pSC101 replicon (i.e., monocopy) and an α-*lac* fragment in which the polylinker of pUC18 is flanked by *Not*I sites. This allows the excision of the cloned DNA segments as *Not*I fragments for further insertion in the single *Not*I site of the transposon vector of pJMS11 (see Fig. 3). Restriction sites: *N*, *Not*I; *E*, *Eco*RI; *Xm*, *Xma*I; *Xb*, *Xba*I; *H*, *Hin*dIII; *C*, *Cla*I; *A*, *Acc*I; *Av*, *Avr*II.

chloramphenicol-resistant vector pCK01 (10), in which a 2.0-kb Ω-*Eco*RI element from pHP45Ω (9) has been inserted into the *Eco*RI site to stop any transcription arising from the *lac* promoter present in the plasmid. pCK02 was cleaved with *Xba*I and *Acc*I and ligated to the 5.8-kb *Xba*I-*Cla*I fragment of pRL4 containing *xylMABN*. The resulting plasmid (pCK03; Fig. 2) was then cleaved with *Xma*I and *Xba*I (thereby releasing the Ω element) to receive a 3.3-kb *Xma*I-*Xba*I fragment of pED3306 containing the *Pu* promoter and the downstream genes *xylUWC* (31). This reconstructed the entire upper TOL pathway downstream of its native promoter, *Pu*, in plasmid pCK04. Two additional restriction sites, *Kpn*I and *Avr*II, were then created at the 5′ end of the operon sequence by insertion of a linker at the single *Xma*I site of the plasmid, thus producing plasmid pCK04AK. In addition, a 2.4-kb DNA segment spanning the regulatory gene *xylR* was prepared as a *Kpn*I fragment by adding *Kpn*I linkers to the ends of the *Hpa*I fragment released upon digestion of plasmid pTK19 (11) with this enzyme. The *Kpn*I segment was then inserted at the single *Kpn*I site of pCK04AK, giving rise to pCK04A*xylR*. This plasmid carries a ca. 12-kb *Not*I insert (named hereafter the *upp* TOL catabolic segment; Fig. 2) with the entire TOL upper pathway, along with its cognate regulatory gene, *xylR*.

The functionality of every enzymatic step of the pathway was verified in vivo as follows. Presentation of indole to *Escherichia coli* cells bearing pCK04A*xylR* (Fig. 2) in the presence of 3-methylbenzyl alcohol resulted in accumulation of a dark-blue precipitate (indigo), which revealed the activity of the enzyme xylene monooxygenase (22), which is encoded by *xylMA* (Fig. 1). When the same strain was exposed to vapors of 3-methylbenzyl alcohol, we observed the transient formation of a product which migrated the same as 3-methylbenzaldehyde in a high-performance liquid chromatography system (data not shown), thus indicating the functionality of the second enzyme of the pathway, benzyl alcohol dehydrogenase (encoded by *xylB*). Finally, pCK04A*xylR* was cotransformed along with the compatible *xylS*$^+$ plasmid pKT570 (20, 21) in the specialized strain *E. coli* CC118*Pm-lacZ*, which bears a chromosomal *lacZ* fusion to the *m*-toluate/XylS-responsive promoter *Pm* (17). In the presence of 3-methylbenzyl alcohol, this strain accumulated high levels of β-galactosidase, i.e., ≥5,000 Miller units (23), which is equivalent to 15-fold induction. This was evidence of the conversion of the aromatic substrate into *m*-toluate by the enzyme benzaldehyde dehydrogenase (encoded by *xylC*) because *m*-toluate activates XylS, which in turn activates the chromosomal *Pm-lacZ* fusion of the host *E. coli* strain (21). These observations indicated that the 12-kb *upp* TOL catabolic segment contains all of the activities of the TOL upper pathway required for the bioconversions of interest (31). Although the products of the *xylUW* (31) and *xylN* (14) genes do not have a known role in the degradation of toluene, they were kept in their original configuration within the upper TOL operon to ensure reliable performance of the system.

Insertion of the hybrid mini-Tn*5* [*upp* TOL] transposon into *P. putida* and excision of the selection marker. Cloning of the *Not*I insert of pCK04A*xylR* spanning the upper TOL operon plus *xylR* (Fig. 2) into the unique *Not*I site of pJMS11 (Fig. 3) gave rise to plasmid pCK05, the organization of which is sketched in Fig. 4. The minitransposon assembled in pJMS11 contains a DNA segment encoding a selection marker (*npt*, which encodes Km^r) and a visual marker (*xylE*, encoding catechol 2,3-dioxygenase). The latter causes colonies to become yellow upon spraying with catechol ($C23O^+$ phenotype). This

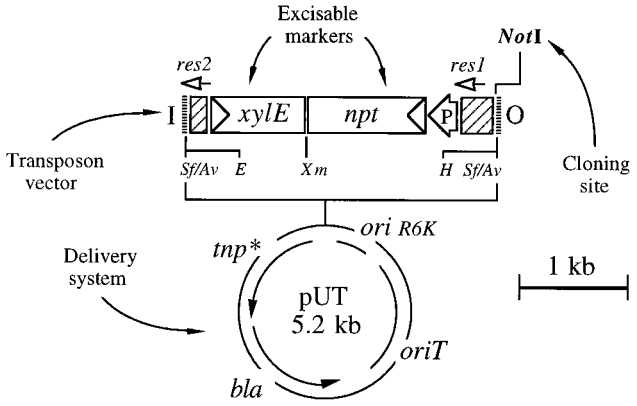

FIG. 3. Organization of pJMS11. This plasmid is the delivery vector for the minitransposon shown at the top and was constructed by deleting the *Not*I insert of pJMS10 (18) and further religation to recover a unique *Not*I cloning site. The suicide donation system (bottom) is that of pUT (7) and includes the Tn*5* transposase gene devoid of *Not*I sites (*tnp**), and Ap^r selection marker (*bla*), an origin of transfer for RP4-mediated mobilization (*oriT*), and the origin of replication of plasmid R6K, which is dependent on the π protein encoded by the *pir* gene carried by specialized λ*pir E. coli* hosts. At the top are the elements included in the mini-Tn*5* transposon vector portion of the plasmid. Besides the single *Not*I site used for cloning of heterologous DNA segments, the predominant feature of this plasmid is the presence of a DNA segment carrying the *xylE* (C23O) and *npt* (Km^r) genes flanked by tandem *res* sites. The arrow-shaped box in front of the *npt* gene indicates the orientation of its promoter (P). Similarly, the triangles in front of the *xylE* and *npt* boxes indicate the orientation of their structural genes. The *res* sequences are represented by the shaded boxes. Cloning of a DNA segment at the single *Not*I site of the plasmid gives rise to a hybrid mobile element flanked by the 19-bp I and O ends of Tn*5*. Restriction sites: *E*, *Eco*RI; *Xm*, *Xma*I; *H*, *Hin*dIII; *Sf*, *Sfi*I; *Av*, *Avr*II.

xylE/*npt* marker segment is flanked by two tandem *res* sequences recruited from the multimer resolution system (*mrs*) of plasmid RP4 (8, 12, 13, 18). The *mrs* system is a site-specific recombination mechanism involving *parA*, a site-specific resolvase that promotes the deletion of any supercoiled DNA placed between two directly oriented *res* sites. The result of this process is excision of the intervening nucleotide sequence

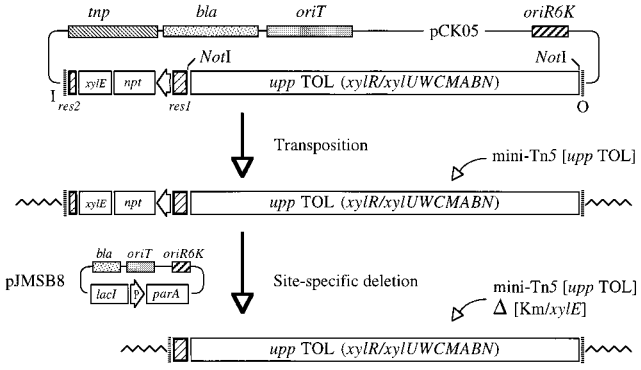

FIG. 4. Insertion of the *upp* TOL catabolic segment into the chromosome of *P. putida* KT2442 and deletion of selection markers. The organization of pCK05, the delivery plasmid for the mobile element mini-Tn*5* [*upp* TOL], is shown at the top (the vector part is not to scale). It includes all of the features of pJMS11 (Fig. 3) plus the *Not*I insert of pCK04A*xylR* (*upp* TOL segment) within the boundaries of the I and O ends of Tn*5*. This plasmid was mobilized to *P. putida* KT2442, and the insertion of the mini-Tn*5* [*upp* TOL] transposon was selected with kanamycin and verified upon spraying of the exconjugants with catechol. In a subsequent step, the Km^r/*xylE* markers were deleted from *P. putida* KT2442::mini-Tn*5* [*upp* TOL] by mobilization of the suicide plasmid pJMSB8 (which bears the *parA* sequence downstream of *lacI*/*Plac*). The final result is stable inheritance of the remainder of the hybrid transposon, i.e., mini-Tn*5* [*upp* TOL] Δ[Km^r/*xylE*].

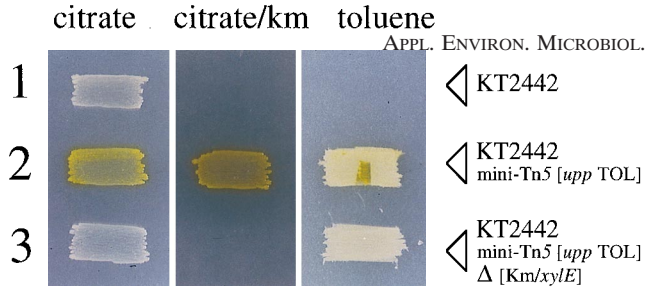

FIG. 5. Phenotypes of *P. putida* KT2442 inserted with the *upp* TOL catabolic segment. Shown is the result of patching three related *P. putida* KT2442 strains in minimal medium with the carbon source and the antibiotic indicated in each case and spraying them with 1% catechol after growth. Strain 1 is *P. putida* KT2442 without any insertion. It can grow only in the medium with citrate and remains white. Strain 2 is *P. putida* KT2442 inserted with the complex transposon mini-Tn*5* [*upp* TOL]. The presence of the *xylE* and *npt* markers in the transposon (Fig. 4) causes the patch to become yellow (C23O$^+$) and to grow in the presence of kanamycin (Km), as well as on toluene, as the only carbon source. Strain 3 is a derivative of strain 2 in which the *xylE*/*npt* portion of mini-Tn*5* [*upp* TOL] has been deleted in vivo. Where indicated, toluene was provided as a saturating vapor phase.

when cells express the cognate resolvase encoded by gene *parA* (8, 18).

On this basis, pCK05 became the delivery plasmid for the mobile element mini-Tn*5* [*upp* TOL]. Insertion of this mini-transposon into the chromosome of *P. putida* KT2442 was carried out by following published protocols (7, 18). Insertions were selected through resistance to kanamycin and verified upon spraying of the exconjugants with catechol. The insertion frequency of mini-Tn*5* [*upp* TOL], defined as the ratio of the number of inserts to the number of recipients (7), was 10^{-5}, a value within the range of other mini-Tn*5* transposons carrying smaller DNA segments (16). Of 50 Km^r and piperacillin-sensitive yellow colonies tested, 30 were able to grow on toluene as the only carbon source (Fig. 5).

Sensitivity to piperacillin ensured the occurrence of authentic transposition events (7), since it rules out the integration of the whole delivery plasmid into the *P. putida* chromosome. The 30 clones with the phenotype caused by insertions of mini-Tn*5* [*upp* TOL] were separately subjected to triparental mating (7) with *E. coli* CC118(pJMSB8) and the helper strain *E. coli* HB101(RK600). The pJMSB8 plasmid is used for transient expression of the RP4 resolvase gene *parA* (18), whereas RK600 assists in the conjugal transfer of pJMSB8 from *E. coli* to *Pseudomonas* (7). To achieve transient expression of *parA*, overnight cultures of *E. coli*(pJMSB8), *P. putida* KT2442:: mini-Tn*5* [*upp* TOL], and *E. coli* HB101(RK600) were separately washed, mixed at 10:1:10, respectively, and then spotted onto the surface of a Luria broth plate. After 4 to 8 h of incubation at 30°C, spots were streaked onto the surface of M9 minimal medium plates with 10 mM citrate as the carbon source (23) but without antibiotics. This allowed growth of *Pseudomonas* but not of the *E. coli* strains present in the mating, which cannot use citrate as a carbon source. The plates were then sprayed with catechol to reveal the loss of the *xylE* marker (along with Km^r). With this procedure, >80% of the colonies had the phenotype anticipated for those losing the *xylE*/*npt* marker segment of the transposon while retaining the ability to grow on toluene as the only carbon source (Fig. 5). Further analysis of the strains by Southern blotting (26) and PCR verified the presence of the entire *upp* TOL element in the chromosome of *P. putida* KT2442 and the loss of the *xylE*/*npt* cassette (data not shown).

Characteristics of *P. putida* derivatives bearing the mini-Tn*5* [*upp* TOL] Δ[Km^r/*xylE*] insert. The strains resulting from the insertion of mini-Tn*5* [*upp* TOL] and subsequent removal of the Km^r/*xylE* segment inherited a new phenotype (growth on toluene due to the acquisition of the *upp* TOL segment) with no other additional markers. Although the concepts for the genetic manipulations were laid out before (18), this work demonstrates their practical utility and its extension in the design of novel bacterial biocatalysts. The recruitment of a DNA fragment to the chromosome of *P. putida* through this procedure resembles the natural mechanisms of insertion and deletion of DNA that occur during natural adaptation processes (15, 27) to such an extent that the constructed strains can be considered quasi-natural. In fact, the final product of the manipulations described here is somewhat reminiscent of that of the naturally occurring genetic event designated "transposition without transposase" by Rappleye and Roth (24). Although in our case, the transposase of Tn*5* is used to effect the insertion of the DNA segment, the *tnp* gene is not inherited by the resulting strain (7, 16). This makes it extremely unlikely that the insertions can be moved again. In this respect, the new DNA segment(s) added to the genome of the recipient strain (in our case, the upper TOL pathway) can be even more stable and predictable than many catabolic genes of natural *Pseudomonas* isolates, the DNA sequences of which are systematically subjected to rearrangements (30). In contrast, no loss of the hybrid mini-Tn*5* insertion was detected after 1,000 generations (data not shown).

The strains bearing mini-Tn*5* [*upp* TOL] Δ[Km^r/*xylE*] grew on toluene as the only carbon source (Fig. 5). The rationale of such a phenotype is the conversion of toluene to benzoate, mediated by the upper TOL enzymes encoded and expressed by the insertion and the further metabolism of benzoate through the housekeeping *ortho*-ring cleavage pathway of the catechol intermediate (Fig. 1). This pathway for degradation of toluene, which avoids a *meta*-cleavage step, is infrequent in natural isolates and will permit us to tackle the biodegradation of difficult chemical species such as chlorotoluenes (1, 2, 19). In a different context, the separate expression of the entire upper TOL pathway away from the *meta* operon should allow bioconversion of an array of substituted toluenes to the corresponding acids (2, 3).

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